Journal of Medical Virology 51:284–289 (1997)

# Amplification of GB Virus-C/Hepatitis G Virus RNA With Primers From Different Regions of the Viral Genome

**J.H. Kao,[1] P.J. Chen,[2] W. Chen,[3] S.C. Hsiang,[3] M.Y. Lai,[2] and D.S. Chen[1,3]***

[1]*Department of Internal Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan*
[2]*Graduate Institute of Clinical Medicine, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan*
[3]*Hepatitis Research Center, National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan*

GB virus-C/hepatitis G virus (GBV-C/HGV) is a newly identified RNA virus. The aim of the study was to compare three primer pairs from the 5′ untranslated region (5′UTR), envelope region 2 (E 2) and nonstructural region 3 (NS 3) of GBV-C/HGV genome for their ability to detect GBV-C/HGV RNA by polymerase chain reaction (PCR) assays. By using PCR with primers from different regions of the viral genome, serum GBV-C/HGV RNA was assayed in 200 at-risk individuals. The sensitivity of this assay was assessed by a titration experiment, and nucleotide sequences of the amplified products were determined directly. Of 200 serum samples, 43 (21.5%) were positive for GBV-C/HGV RNA with at least one of the primer pairs. The positive rates by 5′UTR, NS 3, and E 2 primers were 100%, 98%, and 84%, respectively, and the sensitivity of PCR assays using 5′UTR primers was 10 to 100 times more likely to detect GBV-C/HGV RNA than that of NS 3 and E 2 primers. The average homology of amplified targets to the prototype HGV genome was 89%, 80%, and 85% and the similarity between each amplified target was up to 100%, 90%, and 92% in the 5′UTR, E 2, and NS 3 regions, respectively. Therefore, the 5′UTR of GBV-C/HGV genome is highly conserved and primers deduced from this region can provideva sensitive and specific PCR assay for GBV-C/HGV RNA. ***J. Med. Virol. 51:284–289, 1997.*** © 1997 Wiley-Liss, Inc.

**KEY WORDS:** GB virus-C/hepatitis G virus; reverse transcription-polymerase chain reaction; 5′ untranslated region; envelope region 2; nonstructural region 3



## INTRODUCTION

A group of GB viruses (GBVs) including two independent species (GBV-A, GBV-B) was identified from plasma of a GBV-infected tamarin [Simons et al., 1995b]. A third related virus (GBV-C) was identified in serum from individuals with idiopathic hepatitis [Simons et al., 1995a]. The GBVs are positive-strand RNA viruses with genome sizes of ~9.1 kb to 9.4 kb [Muerhoff et al., 1995; Leary et al., 1996]. The genomic organization of GBVs is similar to those of flaviviruses, and thus resembles that of the hepatitis C virus (HCV) [Leary et al., 1996]. However, phylogenetic analysis showed that these GBVs are not variants of HCV [Simons et al., 1995a; Leary et al., 1996]. It has also been indicated that only GBV-C RNA can be identified in at-risk individuals including patients with non A-E hepatitis [Simons et al., 1995a]. Recently, a flavi-like virus designated hepatitis G virus (HGV) was identified independently from plasma samples of patients with chronic hepatitis [Linnen et al., 1996]. The virus is transmissible by blood transfusion and has a global distribution [Simons et al., 1995a; Linnen et al., 1996]. Alignment of the encoded polyprotein sequences of GBV-C and that of HGV shows amino acid sequence identity at 95% (85% at the nucleotide level) [Zuckerman, 1996]. Thus GBV-C and HGV are variants of the same virus, and this virus is designated GBV-C/HGV at present [Zuckerman, 1996]. Although controversies exist concerning the role of GBV-C/HGV in causing fulminant hepatitis [Yoshiba et al., 1995; Kao et al., 1996a], further studies to clarify the natural history of GBV-C/HGV infection and to ascertain its epidemiol-

*Correspondence to: Dr. D.S. Chen, Hepatitis Research Center, National Taiwan University Hospital, 1 Chang-Te St., Taipei, Taiwan 100.

Received 18 October 1996

TABLE I. Oligonucleotide Primers From Different Regions of GB Virus-C/Hepatitis G Virus (GBV-C/HGV) Genome

| Primer no.[a] | Sequence (5′→3′) | Nucleotide position[b] |
|---|---|---|
| 5′UTR | | |
| 101s | GGCCAAAAGGTGGTGGATGG | 101–120 |
| 350a | GGTCCACGTCGCCCTTCAAT | 331–350 |
| 121s | GTGATGACAGGGTTGGTAGG | 121–140 |
| 330a | GGCAAACGACGCCCACGTAC | 311–330 |
| E 2 | | |
| 1013s | TCTGGAATACCTCTGGAAGG | 1013–1032 |
| 1489a | GCCTCCACCAGTGGTCGCGA | 1470–1489 |
| 1106s | TGAGCAACGGATTGTCATGG | 1106–1125 |
| 1463a | GCGTGTGGGGTTCCGCTTCT | 1444–1463 |
| NS 3 | | |
| 4257s | GACGTGGGCGAGATTCCCTT | 4257–4276 |
| 4494a | GGGTACACTGGAAATTTCG | 4476–4494 |
| 4279s | ATGGGCATGGAATACCCCTCGAGCG | 4279–4303 |
| 4435a | AGTTCTATCATCAAGGA | 4419–4435 |

[a]5′UTR = 5′ untranslated region; E 2 = envelope region 2; NS 3 = nonstructural region 3; s = sense; a = antisense.
[b]Based on the prototype HGV genome [Linnen et al., 1996].

ogy and clinical significance, both in the setting of single infection and in conjunction with other known hepatitis viruses are necessary.

At present, GBV-C/HGV infection can be detected only by reverse transcription-polymerase chain reaction (RT-PCR) with primers deduced from the putative helicase gene within the nonstructural region 3 (NS 3) of viral genome [Simons et al., 1995a]. However, like many other RNA viruses, genetic heterogeneity among GBV-C/HGV strains from different geographic areas indeed exists [Kao et al., 1996b], and thus may result in false-negativity because of primer and template mismatch. Therefore, we compared the efficacy of three oligonucleotide primer pairs derived from either the 5′ untranslated region (5′UTR), the putative structural envelope region 2 (E 2) or the NS 3 of the GBV-C/HGV genome in RT-PCR assays for the detection of serum GBV-C/HGV RNA. The nucleotide sequences of amplified targets from these three regions were examined further to investigate the degree of sequence variability for each region.

## MATERIALS AND METHODS

### Patients

Serum samples were tested from 200 randomly selected individuals who were at increased risk for GBV-C/HGV infection [Simons et al., 1995a; Linnen et al., 1996]. There were 100 patients with chronic HCV infection and 100 polytransfused patients (10 with hemophilia, 20 with aplastic anemia, 30 with thalassemia, and 40 with leukemia). Chronic HCV infection was defined by both positive reactions for second-generation anti-HCV assay (Abbott Laboratories, North Chicago, IL) and presence of serum HCV RNA for at least 6 months. All the serum samples were stored at −70°C until use.

### RNA Extraction and Detection of GBV-C/HGV RNA

The presence of GBV-C/HGV RNA was assayed separately by RT-PCR with nested primers from the 5′UTR, E 2 and NS 3 of the viral genome (Table I). The oligonucleotide primers were synthesized based on the published GBV-C/HGV sequences [Leary et al., 1996; Linnen et al., 1996]. Briefly, RNA was extracted from 100 μl of serum by the single-step acid guanidinium thiocyanate-phenol-chloroform method, then converted into cDNA with random hexamers and Moloney murine leukemia virus reverse transcriptase in a volume of 20 μl. For the first stage PCR, a 25 μl of reaction mixture containing 2 μl of the cDNA sample, 1 × PCR buffer (10 mM tris-HCl pH 9.0, 50mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100), 10 mM of each dNTP, 100 ng of each outer primers (101s and 350a, 1013s and 1489a, and 4257s and 4494a as shown in Table I) and 1 unit of Taq DNA polymerase was amplified in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) for 30 cycles. Each cycle entailed denaturation at 95°C for 60 s, primer annealing at 55°C for 30 sec and extension at 72°C for 60 sec. After the first amplification, 1 μl of the PCR products was reamplified for another 30 cycles with 100 ng of each inner primers (121s and 330a, 1106s and 1463a, and 4279s and 4435a, Table I). The second round of PCR was done in the same manner as the first round. The amplified products were separated in 3% agarose gel electrophoresis and stained by ethidium bromide. To avoid false-positive results, the precautions described by Kwok and Higuchi [1989] were applied to prevent cross contaminations.

The relative sensitivity of the PCR assay was assessed by titration using serial 10-fold dilutions of 10 serum samples known to be positive for GBV-C/HGV RNA. The serum specimens were diluted in uninfected serum before extraction of RNA, and diluted specimens were assayed independently for GBV-C/HGV se-

TABLE II. Positivity of GB Virus-C/Hepatitis G Virus (GBV-C/HGV) RNA by Oligonucleotide Primers From Different Regions of Viral Genome in 7 Patients with Discordant Results

| Patient no. | Sex | Diagnosis | 5′UTR[a] | NS 3[a] | E 2[a] |
|---|---|---|---|---|---|
| 1 | M | Aplastic anemia | + | + | − |
| 2 | M | Aplastic anemia | + | + | − |
| 3 | F | Aplastic anemia | + | + | − |
| 4 | M | Leukemia | + | + | − |
| 5 | M | Leukemia | + | + | − |
| 6 | M | Leukemia | + | + | − |
| 7 | F | Leukemia | + | − | − |

[a]5′UTR = 5′ untranslated region; NS 3 = nonstructural region 3; E 2 = envelope region 2.

quences by using each of the three primer pairs (5′UTR, E 2 and NS 3).

### Sequencing of the Amplified Targets

Nucleotide sequences of the amplified products were directly determined by using fluorescence labelled primers with a 373A Sequencer (Applied Biosystems, Foster City, CA). Sequencing conditions were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). The inner primer pairs 121s and 330a, 1106s and 1463a, and 4279s and 4435a were used as sequencing primers for both directions of the partial 5′UTR, E 2, and NS 3 of GBV-C/HGV genome, respectively.

## RESULTS

Three primer pairs derived from the 5′UTR, E 2 and NS 3 of the viral genome were compared for their efficacy in detecting serum GBV-C/HGV RNA by nested RT-PCR assays. The target sequences amplified were predicted to yield PCR products of 210 bp (5′UTR), 358 bp (E 2), and 157 bp (NS 3). Of 200 high-risk patients, 43 (21.5%) were positive for GBV-C/HGV RNA with primer pairs derived from either 5′UTR, E 2 or NS 3. Among the 43 GBV-C/HGV RNA-positive patients, 36 were positive for all three GBV-C/HGV genome regions and seven showed discordant results (Table II). Overall, the 5′UTR primer pair was most effective in detecting serum GBV-C/HGV RNA, because all viremic patients were detected with this primer pair. On the other hand, the NS 3 and E 2 primer pairs were less effective, with 98% and 84% of the viremic serum samples testing positive, respectively.

The specificity of amplified targets was documented by direct sequencing of the PCR products selected from 10 patients positive for GBV-C/HGV RNA as detected by all of the three primer pairs (Fig. 1). The homology of nucleotide sequences of these amplified targets to the prototype HGV genome was 88%~93% (average 89%), 79%~82% (80%), and 83%~87% (85%) in the corresponding 5′UTR, E 2, and NS 3, respectively [Linnen et al., 1996]. In addition, the homology of nucleotide sequences of the 5′UTR, E 2, and NS 3 between each amplified target ranged from 93% to 100%, 86% to 90%, and 86% to 92%, respectively (Fig. 1).

The relative sensitivity of the PCR assays using the three primer pairs in detecting GBV-C/HGV RNA was assayed in 10 viremic serum samples, and the representative result from a given patient is shown in Table III. The GBV-C/HGV RNA became undetectable in 1: $10^5$, $10^4$, $10^3$ dilution after ethidium bromide staining of the PCR products when the 5′UTR, NS 3, and E 2 primer pairs were used, respectively. Based on these results, the 5′UTR primer pair was 10 to 100 times more sensitive to detect GBV-C/HGV RNA than the NS 3 and E 2 primer pairs in our study.

## DISCUSSION

RNA viruses evolve rapidly with an estimated mutation rate ranging from $10^{-3}$ to $10^{-4}$ base substitutions per genome site per year, and this high mutation rate can be ascribed to an error-prone RNA-dependent RNA polymerase as well as the lack of an associated repair mechanism [Holland et al., 1982]. Thus, many RNA viral genomes exhibit significant genetic heterogeneity as a result of mutations occuring during viral replication. However, the degree of genetic variability varies in different regions of the viral genome because of the different rate of evolution for each region. This fact underlies the difficulty in selecting the ubiquitous oligonucleotide sequences from certain regions as primer pairs for amplification of RNA viruses by RT-PCR.

Two novel flavivirus-like RNA viruses with genome sizes of 9.1 kb to 9.3 kb are independently identified from patients with chronic hepatitis, and are designated GBV-C and HGV, respectively [Simons et al., 1995a; Leary et al., 1996; Linnen et al., 1996]. Both viruses are clearly not variants of HCV by phylogenetic analysis, and a comparison of their putative nonstructural region 3 showed a 100% amino acid identity, suggesting that they are very closely related [Linnen et al., 1996]. Moreover, alignment of the encoded polyprotein sequences of HGV and that of GBV-C shows amino acid sequence identity at 95% (85% at the nucleotide level) [Zuckerman, 1996]. These lines of evidence indicate that HGV and GBV-C are independent isolates of the same virus. Whether this novel blood-borne virus is responsible for a proportion of posttransfusion and community-acquired hepatitis without defined etiology remains to be explored. Because reliable serologic assays for antibodies against GBV-C/HGV are yet to be developed, amplification of GBV-C/HGV RNA sequences by RT-PCR is the only practical method currently available to demonstrate viremia in patients with GBV-C/HGV infection. We recently demonstrated the presence of genetic heterogeneity in GBV-C/HGV strains cloned from different geographic areas, and at least 3 groups of GBV-C/HGV can be identified in Taiwan [Kao et al., 1996b]. Accordingly, the genetic heterogeneity may cause false-negative PCR results because of primer and template mismatch. Thus in the present study we designed primer pairs from three regions (5′UTR, E 2 and NS 3) of the GBV-C/HGV genome and tested their ability to detect GBV-C/HGV in nested RT-PCR assays.

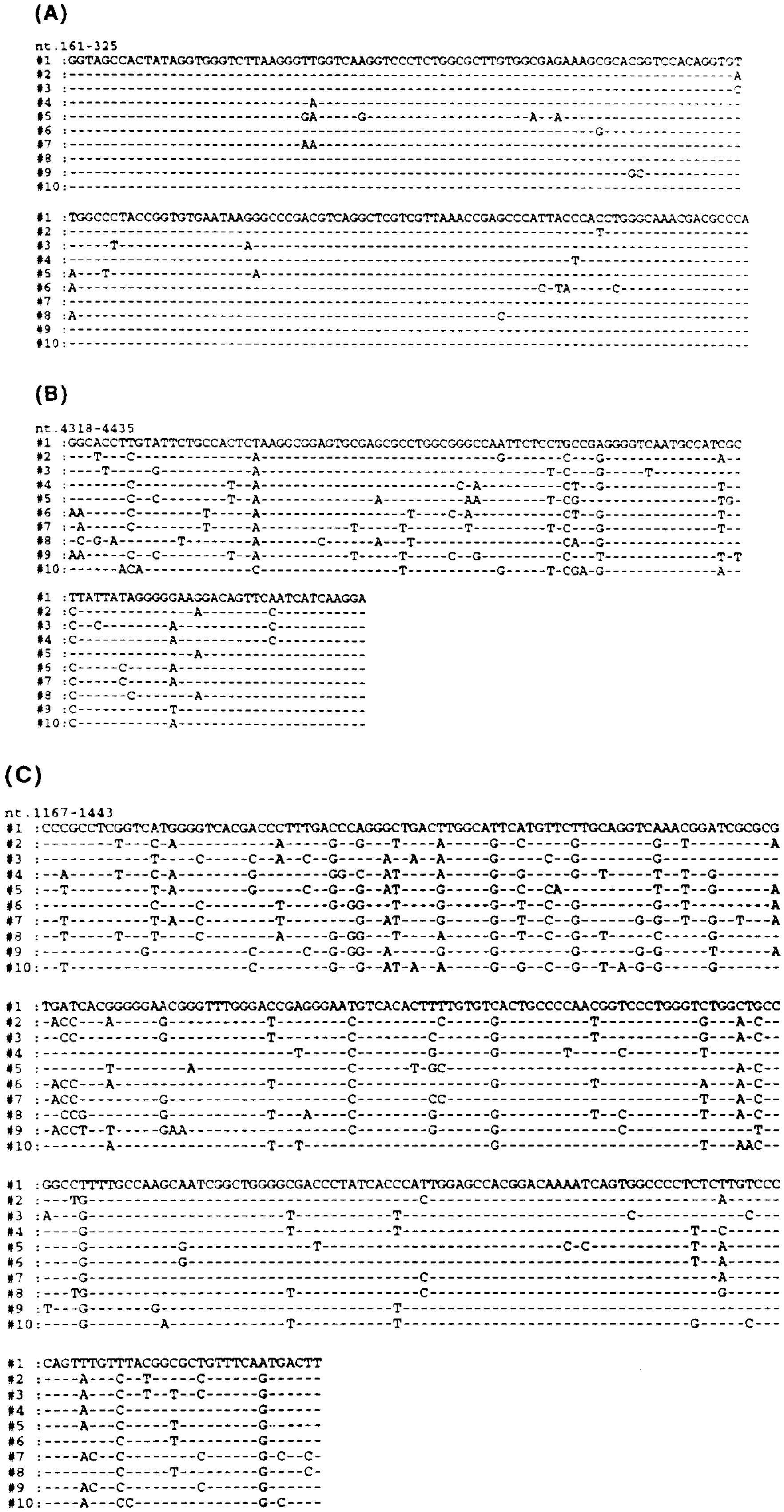


Fig. 1. Alignment of nucleotide sequences of the 5′ untranslated region (5′UTR) **(A),** nonstructural region 3 (NS 3) **(B),** and envelope region 2 (E 2) **(C)** from 10 patients with GBV-C/HGV infection. The nucleotide positions are based on the prototype HGV genome [Linnen et al., 1996]. A dash denotes an identical nucleotide to the first-line sequence. The homology of nucleotide sequences of the 5′UTR, NS 3, and E 2 between each patient ranges from 93% to 100%, 86% to 92%, and 86% to 90%, respectively.

TABLE III. Relative Sensitivity of Oligonucleotide Primers From Different Regions of GB Virus–C/Hepatitis G Virus (GBV–C/HGV) Genome

| | Dilution of serum sample positive for GBV-C/HGV RNA | | | | | |
|---|---|---|---|---|---|---|
| Regions[a] | 1:10 | $1{:}10^2$ | $1{:}10^3$ | $1{:}10^4$ | $1{:}10^5$ | $1{:}10^6$ |
| 5′UTR | +++ | +++ | ++ | + | – | – |
| NS3 | ++ | ++ | + | – | – | – |
| E 2 | ++ | + | – | – | – | – |

[a]5′UTR = 5′ untranslated region; NS 3 = nonstructural region 3; E 2 = envelope region 2.

Our data showed that 43 of 200 (21.5%) high-risk patients were positive for GBV-C/HGV RNA with at least one of the primer pairs derived from different regions of the viral genome. Although the true prevalence may be underestimated because of detecting viremia only in our study, this result was consistent with a previous report that ~18% of chronic hepatitis C, hemophilia, or multiply transfused anemia European patients are GBV-C/HGV RNA-positive [Linnen et al., 1996]. Of the three primer pairs, the 5′UTR primers could detect one more GBV-C/HGV RNA-positive patient than the NS 3 primers (100% vs. 98%) and 7 more such patients than the E 2 primers (100% vs. 84%, $P = 0.054$ by Fisher's exact test). Moreover, the relative sensitivity of PCR assays using the 5′UTR primers was 10 to 100 times more in detection of GBV-C/HGV RNA than that of NS 3 and E 2 primers as demonstrated by a titration experiment with serial dilutions of viremic serum samples (Table III). Although the 5′UTR primers was at least 10 times more sensitive than the NS 3 primers in detecting GBV-C/HGV RNA, their nearly identical detection rates (100% vs. 98%) suggested that most, if not all, patients in the present study had higher serum virus titers. Taken together, these results suggested that primers deduced from the 5′UTR or NS 3 region of the GBV-C/HGV genome are almost equally effective in detecting GBV-C/HGV RNA by PCR assays, however, in patients with low-titer viremia, those deduced from 5′UTR may be more sensitive.

The degree of genetic variability among different regions of the GBV-C/HGV genome remains unknown. We thus sequenced directly the amplified products of 5′UTR, E 2 and NS 3 from 10 GBV-C/HGV RNA-positive patients, and showed that more than 10% nucleotide divergence was found in the 5′UTR and up to 15% and 20% in the NS 3 and E 2, respectively as compared with the prototype HGV genome [Linnen et al., 1996]. In addition, the similarity of nucleotide sequences between each amplified target was up to 100%, 90%, and 92% in the 5′UTR, E 2, and NS 3, respectively (Fig. 1). These data suggested that 5′UTR is a highly conserved part of the GBV-C/HGV genome and may contain invariant domains important in initiation and control of polyprotein translation as in the situation of HCV genome [Okamoto et al., 1990; Houghton et al., 1991; Tsukiya-Kohara et al., 1992; Wang et al., 1993]. This also confirmed the importance of using primer pairs from the more conserved region of the GBV-C/HGV genome to avoid false-negative results caused by mismatch between primer and template resulting from the genetic heterogeneity among GBV-C/HGV strains [Bukh et al., 1992]. In contrast, nucleotide sequences of the E 2 and NS 3 were more variable than those of the 5′UTR. However, whether there exist variable or even hypervariable domains in the envelope regions of GBV-C/HGV genome serving as neutralization epitopes and escaping from host immune pressure by epitope drift, as is seen in HCV [Kao et al., 1995; Weiner et al., 1991, 1992], awaits further studies.

In summary, the 5′UTR of GBV-C/HGV genome is highly conserved and primers deduced from this region can provide a sensitive and specific detection of GBV-C/HGV RNA. Our data also confirm the crucial importance of selecting appropriate primers for the detection of RNA viruses in the serum of infected individuals.

## ACKNOWLEDGMENTS

This study was supported by grants from the Department of Health and the National Science Council, Executive Yuan, ROC.

## REFERENCES

Bukh J, Purcell RH, Miller RH (1992): Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proceedings of the National Academy of Sciences USA 89:187–191.

Holland J, Spindler K, Horodyski F, Grabau E, Nichol S, VandePol S (1982): Rapid evolution of RNA genomes. Science 215:1577–1585.

Houghton M, Weiner A, Han J, Kuo G, Choo QL (1991): Molecular biology of the hepatitis C viruses: Implications for diagnosis, development and control of viral disease. Hepatology 14:381–388.

Kao JH, Chen PJ, Lai MY, Wang TH, Chen DS (1995): Quasispecies of hepatitis C virus and genetic drift of the hypervariable region in chronic type C hepatitis. Journal of Infectious Diseases 172:261–264.

Kao JH, Chen PJ, Chen DS (1996a): GBV-C in the aetiology of fulminant hepatitis. Lancet 347:120.

Kao JH, Chen PJ, Hsiang SC, Chen W, Chen DS (1996b): Phylogenetic analysis of GB virus-C: comparison with isolates among Africa, North America and Taiwan. Journal of Infectious Diseases 174:410–413.

Kwok S, Higuchi R (1989): Avoiding false positive with PCR. Nature 339:237–238.

Leary TP, Muerhoff AS, Simons JN, Pilot-Matias TJ, Erker JC, Chalmers ML, Schlauder GG, Dawson GJ, Desai SM, Mushahwar IK (1996): Sequence and genomic organization of GBV-C: A novel member of the Flaviviridae associated with human non-A-E hepatitis. Journal of Medical Virology 48:60–67.

Linnen J, Wages J, Zhang-Keck ZY, Fry KE, Krawczynski KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih JW, Young L, Piatak M, Hoover C, Fernandez J, Chen S, Zou JC, Morris T, Hyams KC, Ismay S, Lifson JD, Hess G, Foung SKH, Thomas H, Bradley D, Margolis H, Kim JP (1996): Molecular cloning and disease association of hepa-

titis G virus: a transfusion-transmissible agent. Science 271:505–508.

Muerhoff AS, Leary TP, Simons JN, Pilot-Matias TJ, Dawson GJ, Erker JC, Chalmers ML, Schlauder GG, Desai SM, Mushahwar IK (1995): Genomic organization of GB viruses A and B: two new members of the Flaviviridae associated with GB agent hepatitis. Journal of Virology 69:5621–5630.

Okamoto H, Okada S, Sugiyama Y, Tanaka T, Sugai Y, Akahane Y, Machida A, Mishiro S, Yoshizawa H, Miyakawa Y, Mayumi M (1990): Detection of hepatitis C virus RNA by a two-stage polymerase chain reaction with two pairs of primers deduced from the 5′-noncoding region. Japanese Journal of Experimental Medicine 60:215–222.

Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK (1995a): Isolation of novel virus-like sequences associated with human hepatitis. Nature Medicine 1:564–569.

Simons JN, Pilot-Matias TJ, Leary TP, Dawson GJ, Desai SM, Schlauder GG, Muerhoff AS, Erker JC, Buijk SL, Chalmers ML, van Sant CL, Mushahwar IK (1995b): Identification of two flavivirus-like genomes in the GB hepatitis agent. Proceedings of the National Academy of Sciences USA 92:3401–3405.

Tsukiya-Kohara K, Iizuka N, Kohara M, Nomoto A (1992): Internal ribosome entry site within hepatitis C virus RNA. Journal of Virology 66:1476–1483.

Wang C, Sarnow P, Siddiqui A (1993): Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. Journal of Virology 67:3338–3344.

Weiner AJ, Braner M, Rosenblatt J, Richman K, Tung J, Crawford K, Bonino F, Saracco G, Choo QL, Houghton M, Han JH (1991): Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS 1 proteins and the pestivirus envelope glycoproteins. Virology 180:842–848.

Weiner AJ, Geysen HM, Christopherson C, Hall JE, Mason TJ, Saracco G, Bonino F, Crawford K, Marion CD, Crawford KA, Brunetto M, Barr PJ, Miyamura T, McHutchinson J, Houghton M (1992): Evidence for immune selection of hepatitis C virus (HCV) putative envelope glycoprotein variants: potential role in chronic HCV infections. Proceedings of the National Academy of Sciences USA 89:3468–3472.

Yoshiba M, Okamoto H, Mishiro S (1995): Detection of the GBV-C hepatitis virus genome in serum from patients with fulminant hepatitis of unknown aetiology. Lancet 346:1131–1132.

Zuckerman AJ (1996): Alphabet of hepatitis viruses. Lancet 347:558–559.